

Thesis for doctoral degree (PhD)

February, 2019

**Development of an Efficient Test for Autoimmune disease
using Gold Nanoparticles**



Anantdeep Kaur

Faculty of Science

15 Broadway, Ultimo NSW 2007

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Anantdeep Kaur declare that this thesis, is submitted in fulfilment of the requirements for the award of PhD degree, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution. This research is supported by an Australian Government Research Training Program Scholarship.

Anantdeep Kaur

Production Note:

Signature removed prior to publication.

15/2/2019

ACKNOWLEDGEMENTS

Foremost I would like to express my sincere gratitude to my supervisor's Dr Olga Shimoni and Prof Michael Wallach for believing in me and giving me the opportunity to work on this project. It has been a great learning experience that was made possible by their continuous encouragement and support. I will forever be grateful to them for their continuous support and guidance. I'm thankful to you for being such a wonderful mentors for guiding me through the research.

I would also like to thank Dr Jason Tye-Din, Chair Coeliac Australia, MBBS, PhD, FRACP, Immunology Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia, for providing clinical samples as well for his constant support, guidance and words of wisdom. I am thankful for his suggestions and guidance during the course of the study. I am thankful to the University of Technology Sydney Research Ethics committee for providing ethical approval for the clinical research.

The time I have spent in lab with such a wonderful and kind team has left me with very fond memories of my PhD study. I would like to express my sincere thanks to Ying, David, Alex, Yvonne, Wei, Jacquiline for promoting a stimulating and welcoming social environment in the lab and for their continuous support and kind words. I am also thankful to Buket Demrici for her support and encouragement.

My sincere thanks to my family for their extended support throughout this journey. My parents, my brother have given me their unequivocal support throughout, as always, for which mere expression of thanks likewise does not suffice. I am thankful to you for believing in me and providing me with this beautiful chance to make my dreams come true.

Last, but by no means least, I would like to acknowledge the financial support of the Australian Government and the Science Faculty at UTS, particularly in the award of an Australian Government Research Training Program Scholarship and travel grants that provided necessary financial support for this project.

List of patents and papers

Patent Entitled: Nanoparticles adsorbed with gliadin molecules

Patent date Issued Feb 15, 2018

Patent issuer and number: AU International Patent Application PCT/AU2018/050125. This work has been published with International Publication No. WO 2018/148801.

Kaur, A., Shimoni, O. & Wallach, M. 2017, 'Coeliac disease: from etiological factors to evolving diagnostic approaches', *Journal of Gastroenterology*, vol. 52 (9), pp. 1001–1012. <https://doi.org/10.1007/s00535-017-1357-7>*.

Kaur, A., Wallach, M. & Shimoni, O. 2018, 'A novel diagnostic test for coeliac disease using gliadin coated gold nanoparticles' (under review).

Kaur, A., Shimoni, O. & Wallach, M. 2018, 'A novel screening test for coeliac disease using peptide functionalised gold nanoparticles', *World Journal of Gastroenterology*, vol. 24(47), pp. 5379-5390*.

* Papers reprinted with permission from Publishers

Statement of contribution of authors

Paper I: Coeliac disease: from etiological factors to evolving diagnostic approaches

Author contributions: I (Anantdeep Kaur) researched and reviewed the literature and analysed data. I (Anantdeep Kaur) wrote the paper with critical revisions related to the intellectual content of the manuscript from Prof Michael Wallach and Dr Olga Shimoni.

Paper II: Novel diagnostic assay for coeliac disease using gliadin coated gold nanoparticles

Author contributions: Dr Olga Shimoni and Prof Michael Wallach designed research, I (Anantdeep Kaur) performed research and analysed data. I (Anantdeep Kaur) wrote the paper with critical revisions related to the intellectual content of the manuscript from Prof Michael Wallach and Dr Olga Shimoni.

Paper III: A novel screening test for coeliac disease using peptide functionalised gold nanoparticles

Author contributions: Dr Olga Shimoni and Prof Michael Wallach designed research, I (Anantdeep Kaur) performed research and analysed data. I (Anantdeep Kaur) wrote the paper with critical revisions related to the intellectual content of the manuscript from Prof Michael Wallach and Dr Olga Shimoni.

Awards and Prizes

UTS Academic Excellence Grant, 2013

University of Technology Sydney

The Australian Government Research Training Program Scholarship, 2015

Department of Industry, Innovation Science, Research and Tertiary Education

Australian Government

Runner up UTS Science 3 Minute Thesis (3MT) Competition, 2017

3MT Topic: ‘To go gluten free or not?’

University of Technology Sydney

Table of Contents

1. Chapter I

Paper I: Coeliac disease: from etiological factors to evolving diagnostic approaches

1.1	General introduction to coeliac disease	2
1.2	CD Prevalence	3
1.3	CD: Iceberg Model	5
1.4	CD pathology and histology	6
1.5	Clinical Presentations of CD	8
1.5.1	Classical (Typical) Form	8
1.5.2	Atypical Form	9
1.5.3	Asymptomatic (Silent) Form.....	11
1.5.4	Undiagnosed, Potential coeliac.....	11
1.5.5	High at-risk persons.....	12
1.6	Role of α -Gliadin as the trigger for CD.....	13
1.6.1	γ -Gliadins	14
1.7	Pathogenesis of CD	15
1.7.1	Activation of Immune Response	16
1.7.2	Role of Intra-epithelial lymphocytes in CD	18
1.7.3	Role of tissue transglutaminase enzyme in CD	19
1.7.4	Release of auto-antibodies in CD	21
1.7.5	Coeliac Toxicity and amino acid composition of gliadin	22
1.8	Diagnosis for CD	23

1.8.1	Intestinal Biopsy for testing CD	24
1.8.2	Genetic testing for CD	25
1.9	Detection of antibodies useful in the diagnosis of CD	27
1.9.1	Anti-Gliadin Antibody (Anti-AGA)	28
1.9.2	Anti-Endomysial Antibodies (Anti-EMA)	30
1.9.3	Anti-Transglutaminase Antibodies (Anti-tTG)	31
1.9.4	Anti-Deamidated Gliadin Peptide (Anti-DGP) antibodies	32
1.9.5	Anti-Synapsin and Anti-Ganglioside antibodies	34
1.10	Diagnosis based on Gluten-specific T cells	35
1.11	Saliva tests for CD	37
1.11.1	Salivary Anti-gliadin antibodies for diagnosing CD	38
1.12	The challenges in the development of a commercial kit for	40
	diagnosing CD	
1.13	Outlook for the future development of new diagnostic tools	43
	for CD	
1.14	CD treatment and management.....	44

2. Chapter II

Background on the nanoparticles

2.1	Gold Nanoparticles	46
2.2	Properties of Gold Nanoparticles	47
2.3	Optical properties of gold nanoparticles for sensing applications	48
2.4	SPR-based biosensors	49
2.5	Gold nanoparticles as SPR biosensors	50
2.6	Protein adsorption to nanoparticle surface	51
2.7	Colorimetric sensing using gold nanoparticles	54

2.7.1	Detection of metal ions	54
2.7.2	Detection of proteins	55
2.8	Aims and approaches of this project	58

3 Chapter III

Materials and methods

3.1	Reagents	60
3.1.1	Peptide	60
3.1.2	Clinical samples for assay validation	61
3.2	Alpha-gliadin derived from <i>Triticum aestivum</i>	61
3.3	Coating of gliadin on AuNP surface	62
3.4	Coating of peptide sequence to AuNP using Avidin-Biotin interaction	63
3.5	Calculation of molar extinction coefficient of gliadin, BSA and peptide ...	64
3.6	Titration procedure to find amount of protein needed to saturate and stabilise the colloidal gold	65
3.7	Gliadin solubilisation in solvents	66
3.8	Validation of gliadin protein solubilisation protocol	67
3.9	Preparation of the AuNPs coated with gliadin protein	70
3.9.1	Determination of suitable conditions to coat gliadin on the surface of AuNPs	72
3.10	Preparation of the AuNPs coated with Bovine Serum Albumin (B.S.A)	73
3.11	Preparation of AuNPs coated with peptide sequence.....	74
3.12	Characterisation of the protein and peptide coated AuNPs	77
3.13	Antibody Titration	82

3.14	Antigen-Antibody Interactions	85
3.15	Antibody Specificity	88
3.16	Hypothesis of the study	88
3.17	Analysis of the interaction of gliadin-AuNP with anti-gliadin antibody and non-specific IgG antibody	90
3.18	Analysis of the interaction of AuNPs coated with peptide with anti-gliadin antibody and non-specific IgG antibody	91
3.19	Analysis of the interaction of gliadin-AuNPs with anti-gliadin antibodies in human saliva	92
3.20	Analysis of the interaction of gliadin-AuNPs with anti-gliadin antibodies in human serum	92
3.21	Analysis of the interaction of peptide-AuNPs with anti-gliadin antibodies in human serum	93
3.22	Analysis for Anti-gliadin antibody in clinical human serum	94
3.23	Concentration of immunoglobulins in clinical human serum	94
3.24	Desalting of concentrated immunoglobulins	95
3.25	Analysis for Anti-gliadin antibody in concentrated clinical human serum ...	96
3.26	Composition of the patient sample cohort assessed for assay validation	96
3.27	Determination of Immunoassay sensitivity	102
3.27.1	Colorimetric Response calculation for clinical sample analysis	106
3.28	Statistical Analysis	104

4 Chapter IV

Paper II: Novel diagnostic assay for coeliac disease using gliadin coated gold nanoparticles

4.1	Background	107
-----	------------------	-----

4.2	Introduction	108
4.3	Materials and Methods	111
4.3.1	Reagents	111
4.3.2	Preparation of the AuNPs coated with gliadin protein	111
4.3.3	Preparation of the AuNPs coated with BSA	112
4.3.4	Determination of the concentration of gliadin and BSA coated AuNPs	112
4.3.5	Assay for AGA	113
4.3.6	Colorimetric Response Curve	116
4.4	Results and Discussion	117
4.4.1	Incubation of gliadin-coated AuNPs with AGA	119
4.4.2	Testing AGA in spiked serum	123
4.4.3	Anti-gliadin antibody binding to gliadin-AuNPs in saliva	126
4.4.4	Testing clinical samples	127
4.5	Conclusions	134

5 Chapter V

Paper III: A novel screening test for coeliac disease using peptide functionalised gold nanoparticles

5.1	Background	136
5.2	Introduction	137
5.3	Materials and Method	138
5.3.1	Reagents	138
5.3.2	Peptide	139
5.3.3	Preparation of the AuNPs coated with NeutrAvidin	139
5.3.4	Preparation of the AuNPs coated with peptide using linker	140
5.3.5	AGA assay	141

5.3.6	Colorimetric Response Curve	144
5.4	Results and Discussion	144
5.4.1	Incubation of Peptide coated AuNPs with AGA	149
5.4.2	Testing AGA in spiked serum	154
5.4.3	Testing clinical samples	156
5.5	Conclusions	162

6 Chapter VI

Conclusions of the study

6.1	CD diagnostic assay using gliadin and peptide coated AuNPs	165
6.2	Summary and Future Work	168

Bibliography

List of Tables and Figures

TABLE	DESCRIPTION
1	Histological scoring system for CD
2	Comparison of different histological scoring systems for CD
3	Major clinical manifestations of CD in children and adults
4	Disorders associated with CD
5	Some of the reported anti-gliadin antibody (AGA) testing for screening CD.
6	Some of the reported anti-Transglutaminase antibody (anti-tTG) testing results for screening CD.
7	Some of the reported anti-deamidated gliadin peptide antibody (Anti-DGP) testing for screening CD.
8	Calculated molar absorption coefficients of gliadin, BSA and peptide
9	Average protein concentration (mg/mL) of gliadin in sample with and without the addition of IPA.
10	Range of AuNP: gliadin ratios
11	No. of moles of gold nanoparticles used in the analysis
12	No. of moles of gliadin, peptide, Maleimide-PEG ₁₁ -Biotin and NeutrAvidin and used in the analysis
13	No. of moles of Anti-gliadin antibody used in the analysis
14	Serology levels of active coeliac sufferers in the clinical sample cohort based on the histology and tTG (Tissue transglutaminase), DGP (Deamidated gliadin peptides) results are indicated as IgA or IgG levels followed by normal reference ranges in brackets.
15	Serology levels of undiagnosed coeliac sufferers in the clinical sample cohort based on the histology and tTG (Tissue transglutaminase), DGP (Deamidated gliadin peptides) results are indicated as IgA or IgG levels followed by normal reference ranges in brackets.
16	Serology levels of potential coeliac sufferers in the clinical sample cohort based on the histology and tTG (Tissue transglutaminase), DGP (Deamidated gliadin peptides) results are indicated as IgA or IgG levels followed by normal reference ranges in brackets.
17	Serology levels of coeliac sufferers with T1DM in the clinical sample cohort based on the histology and tTG (Tissue transglutaminase), DGP (Deamidated gliadin peptides) results are indicated as IgA or IgG levels followed by normal reference ranges in brackets.

18	Serology levels of treated coeliac sufferers in the clinical sample cohort based on the histology and tTG (Tissue transglutaminase), DGP (Deamidated gliadin peptides) results are indicated as IgA or IgG levels followed by normal reference ranges in brackets.
19	Serology levels of non-coeliac individuals in the clinical sample cohort based on the histology and tTG (Tissue transglutaminase), DGP (Deamidated gliadin peptides) results are indicated as IgA or IgG levels followed by normal reference ranges in brackets.
CHAPTER IV	
20	Calculated p-value in AuNP coated with gliadin in the presence of AGA antibody and the control antibody (IgG from rabbit serum) at dilutions 2-10 µg/mL.
21	Comparison of the patient samples analysed using the AuNP-AGA test with the previously existing histology and serological results.
22	Analysis of 7 samples with potential or undiagnosed CD using the AuNP-AGA test as compared with previously existing serology.
CHAPTER V	
23	Shows the calculated p-value in AuNP coated with peptide in the presence of AGA antibody and the control antibody (IgG from rabbit serum) at dilutions 2-20 µg/mL.
24	Comparison of the patient samples analysis using the AuNP-Peptide-AGA test with previously existing histology and serological results.
25	Analysis of 7 samples with potential or undiagnosed CD using the Peptide-AuNP-AGA test as compared with previously existing serology.

FIGURE	DESCRIPTION
1	Pathological features of CD
2	The general structure of α -Gliadin protein
3	Schematic diagram showing the pathway for release of anti-gliadin and anti-transglutaminase antibodies.
4	Gliadin modifications by enzyme tissue transglutaminase (tTG) (adapted).
5	Role of tTG in the progression of mucosal damage in CD
6	FASTA sequence of alpha-gliadin
7	CD diagnostic algorithm
8	Representation of surface plasmon resonance for gold nanoparticles
9	Representation of Avidin-Biotin interactions
10	Three step protocol for solubilisation of gliadin using a surfactant, small chain alcohol and heating
11	Average protein concentration (mg/mL) of gliadin at different temperatures (20°C to 80°C).
12	Schematic representation of the surface modification of AuNPs with gliadin
13	Schematic representation of the surface modification of AuNPs with BSA.
14	Schematic representation of the surface modification of AuNPs with NeutrAvidin.
15	The preparation of Maleimide-PEG ₁₁ -Biotin-peptide molecule.
16	Schematic showing the preparation of the AuNPs coated with peptide using linker molecule.
17	UV-Vis spectra of 20 nm gold nanoparticles at 525 nm.
18	3-D structure of hydrophobic and globular protein gliadin.
19	Secondary structure of hydrophobic and globular protein gliadin.
20	Dimensional representation of interactions of AuNP coated with gliadin and IgG antibody.
21	Representation of specificity of antigen-antibody interactions.
22	Representation of specificity of antigen-antibody interactions hypothesis.
23	Schematic showing the concentration of immunoglobulins using saturated ammonium sulphate solution.
24	Colorimetric response curve plotted in AuNP coated with gliadin.
CHAPTER IV	
25	Characterisation of gliadin coated AuNPs
26	Testing gliadin-coated AuNPs with AGA
	xv

27	Incubation of uncoated AuNPs in serum with AGA at various dilutions.
28	Incubation of BSA coated AuNPs in serum with AGA at various dilutions.
29	Detection of AGA in spiked human serum using gliadin-coated AuNPs.
30	Colorimetric response curve plotted in AuNP coated with gliadin in 1:10 diluted serum following the addition of AGA antibody at dilutions 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL and 10 µg/mL.
31	Colorimetric response curve plotted in AuNP coated with gliadin in 1:50 diluted serum following the addition of AGA at dilutions 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL and 10 µg/mL.
32	Testing gliadin coated AuNPs with AGA in saliva
33	Representation of the distribution of clinical samples using AuNP-AGA Assay
CHAPTER V	
34	Schematic representation of preparation of peptide coated AuNPs.
35	Characterisation of peptide coated AuNPs
36	Characterisation of AuNP coated with NeutrAvidin using DLS that showed an increase in the hydrodynamic size of the uncoated vs coated particles from 20 nm to 25 nm respectively.
37	Testing peptide-coated AuNPs with AGA
38	Incubation of AuNPs coated with peptide with AGA at various dilutions
39	Incubation of AuNPs coated with peptide with control antibody at various dilutions.
40	Detection of AGA in spiked human serum using peptide-coated AuNPs
41	Representation of the distribution of clinical samples using AuNP-Peptide-AGA test

List of Abbreviations

CD	Coeliac Disease
AGA	Anti-gliadin antibody
RCD	Refractory Coeliac Disease
HLA	Human Leukocyte Antigen
MHC	Major Histocompatibility Complex
TG2	Tissue Transglutaminase
FADD	Fas associated via death domain
DED	Death Effector Domain
IL	Interleukin
NK	Natural killer
TCR	T cell receptor
LMW	Low molecular weight
CDR	Complementarity determining region
DGP	Deamidated gliadin peptides
Ig	Immunoglobulin
Anti-EMA	Anti-endomysial antibodies
Anti-tTG	Anti-transglutaminase antibodies
Anti-DGP	Anti-Deamidated gliadin peptide
MMR	Mumps and Rubella vaccine
HAV	Hepatitis A Virus
EGF	Epidermal Growth Factor
NCGS	Non Coeliac Gluten Sensitivity
IFA	Indirect immunofluorescence

RIA	Radioimmunoassay
GFD	Gluten free diet
SPR	Surface plasmon resonance
DIG	Diffusion in gel
IBD	Inflammatory bowel disease
GIP	Gluten immunogenic peptides
AuNP	Gold Nanoparticle
LSPR	Localised Surface Plasmon Resonance
PEG	Polyethylene glycol
ELISA	Enzyme linked immunosorbent assay
HCG	Human Chorionic Gonadotrophin
Mab	Monoclonal antibody
CTAB	Cetyl trimethylammonium bromide
IPA	Isopropyl Alcohol
BSA	Bovine Serum Albumin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PBS	Phosphate Buffer Saline
DLS	Dynamic Light Scattering
°C	Celsius
Å	Angstrom
kDa	kiloDalton

ABSTRACT

Coeliac disease results because of an unusual immune response to the digestion of gliadin protein. Patients suffering from the disease show varying degrees of chronic inflammation within the small intestine ranging from mild lesions to completely flat mucosa. The immune response in turn, leads to the production of a number of cytokines and antibodies that are linked to the disease and its pathological effects. The released antibodies can be used as specific biomarkers for developing reliable diagnostic tests. Over the years, different approaches like the use of mucosal biopsy, genetic typing of the disease associated gene, gliadin induced cytokines, antibodies and auto-antibodies have been used to develop diagnostic tests for coeliac disease. However, in spite of the encouraging initial results in terms of sensitivity and specificity, the existing tests have limited scope as point-of-care tests.

The current study was aimed at developing a coeliac diagnostic assay based on the properties of the gold nanoparticles combined with the specificity of the antibodies from serum as well as saliva. In this study, I developed a novel diagnostic test for coeliac disease based on the coating of gold nanoparticles with gliadin, the highly antigenic protein responsible for inducing the symptoms of coeliac disease. A novel protocol for binding the hydrophobic gliadin protein on the surface of the gold nanoparticles was developed in this study. This was followed by the development of a simple, single antibody serology based diagnostic test.

Finally, I used the assay on thirty patient serum samples in a blinded assessment and compared the results with the data from previously run serological and pathological tests on these patients. When tested on real patient samples, the data showed that the developed assay had an overall accuracy of over 96%.

Furthermore, I developed an assay based on the coating of gold nanoparticles with a peptide sequence derived from gliadin. To develop the serological assay based on the peptide functionalised nanoparticles, I first established a stable suspension of peptide coated gold nanoparticles and then tested the assay on spiked serum samples. I then used the assay to test thirty patient serum samples and found that the peptide functionalised nanoparticle-based assay could distinguish coeliac disease from non-coeliac disease patients with an accuracy of 86.6%.

This study demonstrates the potential of gold nanoparticle-based approach to be adapted for developing a point-of-care screening assay for diagnosis for CD. The developed assay could be a part of an exclusion based diagnostic strategy and prove beneficial for testing high coeliac disease risk populations.